MOL PHARM

MOLECULAR PHARMACOLOGY

Itraconazole-Induced Cholestasis: Involvement of the Inhibition of Bile Canalicular Phospholipid Translocator MDR3/ABCB4 [S]

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ABSTRACT

Biliary secretion of bile acids and phospholipids, both of which are essential components of biliary micelles, are mediated by the bile salt export pump (BSEP/ABCB11) and multidrug resistance 3 P-glycoprotein (MDR3/ABCB4), respectively, and their genetic dysfunction leads to the acquisition of severe cholestatic diseases. In the present study, we found two patients with itraconazole (ITZ)-induced cholestatic liver injury with markedly high serum ITZ concentrations. To characterize the effect of ITZ on bile formation in vivo, biliary bile acids and phospholipids were analyzed in ITZ-treated rats, and it was revealed that biliary phospholipids, rather than bile acids, were drastically reduced in the presence of clinically relevant concentrations of ITZ. Moreover, by using MDR3-expressing LLC-PK1 cells, we found that MDR3-mediated efflux of [14C]phosphatidylcholine was significantly reduced by ITZ. In contrast, BSEP-mediated transport of [3H]taurocholate was not significantly affected by ITZ, which is consistent with our in vivo observations. In conclusion, this study suggests the involvement of the inhibition of MDR3-mediated biliary phospholipids secretion in ITZ-induced cholestasis. Our approach may be useful for analyzing mechanisms of drug-induced cholestasis and evaluating the cholestatic potential of clinically used drugs and drug candidates.

Introduction

Bile formation is one of the most essential functions of the liver, and several ATP-binding cassette (ABC) transporters are known to be involved in the biliary secretion of biliary lipids, organic solutes, and xenobiotics (Oude Elferink and Paulusma, 2007). Among them, the bile salt export pump (BSEP/ABCB11) and multidrug resistance 3 P-glycoprotein

(MDR3/ABCB4) have been recognized to play particularly crucial roles in the physiological formation of bile, because genetic dysfunction of each of these two transporters causes a severe hereditary disorder, progressive familial intrahepatic cholestasis (PFIC). BSEP is expressed on the bile canalicular membrane of hepatocytes and functions as an exporter of bile acids into bile (Gerloff et al., 1998; Noé et al., 2002), and its dysfunction leads to the intrahepatic accumulation of bile acids resulting in the acquisition of the severe liver damage. MDR3 mediates the translocation of phospholipids from the inner to outer leaflet of bile canalicular membranes (Smit et al., 1993; van Helvoort et al., 1996), and the translocated phospholipids are necessary to form biliary micelles with bile acids. Without the secretion of phospholipids,

ABBREVIATIONS: ABC, ATP-binding cassette; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BSEP, bile salt export pump; DILI, drug-induced liver injury; EGFP, enhanced green fluorescent protein; γ-GTP, γ-glutamyl transpeptidase; ITZ, itraconazole; LFT, liver function test; LLC-BSEP/NTCP, bile salt export pump- and Na⁺/taurocholate cotransporting polypeptide-expressing LLC-PK1 cell; LLC-EGFP, enhanced green fluorescent protein-expressing LLC-PK1 cell; LLC-EGFP/NTCP, enhanced green fluorescent protein- and Na⁺/taurocholate cotransporting polypeptide-expressing LLC-PK1 cell; LLC-MDR3, multidrug resistance 3 P-glycoprotein-expressing LLC-PK1 cell; MDR3, multidrug resistance 3 P-glycoprotein; MOI, multiplicity of infection; NTCP, Na⁺/taurocholate cotransporting polypeptide; PC, phosphatidylcholine; PFIC, progressive familial intrahepatic cholestasis; PS, permeability-surface area product; ANOVA, analysis of variance; HP-β-CD, hydroxypropyl-β-cyclodextrin; UPLC-MS/MS, ultraperformance liquid chromatography/tandem mass spectrometry.

hepatocytes and cells lining the biliary tract suffer from the detergent effect of bile acids in bile.

Considering the physiological significance of BSEP and MDR3, drug-mediated functional disruption of these transporters may result in drug-induced cholestasis, which is one form of drug-induced liver injury (DILI) and is characterized by elevations of alkaline phosphatase (ALP), serum γ-glutamyl transpeptidase (γ-GTP), and total bilirubin. Indeed, several cholestatic drugs are known to inhibit BSEP-mediated transport of bile acids in vitro (Stieger et al., 2000; Fattinger et al., 2001; Funk et al., 2001; Wang et al., 2001; Bode et al., 2002; Horikawa et al., 2003; Vallejo et al., 2006), although their inhibitory effects have been usually observed at much higher concentrations than those seen in clinical situations. In addition, serum γ-GTP levels generally remain normal in BSEP-related genetic cholestasis (PFIC2), whereas serum levels of this enzyme are markedly elevated in MDR3-related genetic cholestasis (PFIC3). These pieces of information suggest that some drug-induced cholestasis may occur by the inhibition of MDR3 rather than BSEP.

Concerning the clinically observed drug-induced cholestasis, an antifungal agent, itraconazole (ITZ), has been reported to cause cholestatic liver injuries (Lavrijsen et al., 1992; Hann et al., 1993; Gallardo-Quesada et al., 1995; Srebrnik et al., 2005). In addition, the U.S. Food and Drug Administration has received reports of at least 24 cases of DILI, including 11 deaths associated with the use of ITZ (Song and Deresinski, 2005). In the present study, we also observed marked elevations of serum ALP, γ-GTP, and total bilirubin levels in two patients who received the administration of ITZ. Because serum concentrations of ITZ in these patients were remarkably higher than the concentrations reported in a population pharmacokinetics study of Japanese patients (Yamaguchi et al., 2006), we suspected that the high concentrations of ITZ in the liver might inhibit bile formation and increase the risk of cholestasis. To verify this hypothesis, we analyzed the biliary secretion of bile acids and phospholipids in ITZ-administered rats in combination with in vitro experiments for the inhibitory effect of ITZ on human MDR3- and BSEP-mediated transport.

Materials and Methods

Materials. ITZ solution (itrizole injection 1%) was purchased from Janssen Pharmaceutical K.K. (Tokyo, Japan). [^{14}C]Choline (55 mCi/mmol) was purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO), and [^{3}H]taurocholate (4.6 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). LLC-PK1 cells were purchased from the American Type Culture Collection (Manassas, VA) and cultured in Medium 199 (Sigma-Aldrich, St. Louis, MO) with 10% fetal bovine serum (GIBCO, Carlsbad, CA) and 1% penicillin/streptomycin (Nacalai Tesque, Kyoto, Japan) at 37°C in an atmosphere supplemented with 5% CO$_2$. All other chemicals used were commercially available and of reagent grade.

Patient Information. The protocol of the present clinical study was approved by the Institutional Ethics Committee of the University of Tokyo and conducted in accordance with the ethical guidelines of the Declaration of Helsinki. Twenty-four inpatients in the University of Tokyo Hospital, whose written informed consent was obtained from October 2007 to December 2008, received a diagnosis of DILI; for these patients, possibilities of other common causes of hepatic injury (such as liver carcinoma, viral hepatitis, gallstone, and primary biliary cirrhosis) were excluded. Among them, a 67-year-old

woman (patient 1), a 43-year-old man (patient 2), and a 36-year-old woman (patient 3) exhibited elevations in liver function tests (LFTs) during the administration of ITZ (once-daily dosing of 200 mg as oral solution) and after discontinuing the administration, LFTs returned to normal. In the present study, serum ITZ concentrations in these patients were determined using the serum specimens remained after routine laboratory analyses for clinical purposes. DNA microarray analyses using the patients' genomic specimens were performed with DMET Plus Premier Pack (Affymetrix, Santa Clara, CA) according to the manufacturer's protocol.

Determination of Serum ITZ Concentration. To measure the serum concentrations of ITZ, serum specimens were mixed with 5 volumes of methanol and centrifuged at 20,000g for 15 min. Supernatants were used for the analysis using an ACQUITY UPLC-Quattro Premier XE (Waters Corporation, Milford, MA), an ultraperformance liquid chromatography coupled with an electrospray ionization tandem mass spectrometry system (UPLC-MS/MS). The column used was a Waters UPLC BEH C18 Shield (3 μm; 2 × 100 mm), and UPLC was performed under gradient mobile phase conditions using a mixture of 0.1% formic acid in water and 0.1% formic acid in acetonitrile as solvents [0.3 ml/min; 0–1.0 min, 95:5 (v/v) 1.0–7.0 min, 95:5 (v/v) to 5:95 (v/v); 7.0–9.0 min, 5:95 (v/v) to 95:5 (v/v), 9.0–10.0 min, 95:5 (v/v)]. The UPLC eluent was introduced into a mass spectrometer via positive electrospray ionization mode. Quantification of ITZ was performed in multiple reactions monitoring mode (705.14 > 392.30; cone, 60 V; collision, 36 eV) using lovastatin as the internal standard (405.25 > 173.10; cone, 20 V; collision, 22 eV).

In Vivo Experiments. Animal studies were performed according to the method approved by the Institutional Animal Care Committee of the University of Tokyo. All animals received human care according to the criteria outlined in the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996).

Male Sprague-Dawley rats (Japan SLC, Shizuoka, Japan) weighing 210 to 250 g were fed standard rat chow and water. Before operation, rats were anesthetized with an intraperitoneal injection of 1.25 g/kg urethane (Sigma-Aldrich). The femoral artery and vein were cannulated with 0.5 mm polyethylene tube and bile duct with 0.2 mm polyethylene tube (both from Natsume, Tokyo, Japan). Then, rats received 10 mg/ml ITZ solution intravenously [containing 0.40 g/ml hydroxypropyl-β-cyclodextrin (HP-β-CD) and 25 μl/ml propylene glycol] as a bolus dose of 43 mg/kg and then infused with 3.3 mg/ml ITZ solution (containing 0.13 g/ml HP-β-CD and 8.3 μl/ml propylene glycol) for 2 h at a rate of 7.3 mg/kg/h (0.5 ml/h). Bile and blood specimens were taken every 30 min, and liver samples were collected after euthanasia. Plasma specimens were obtained by centrifuging blood specimens at 1000g for 15 min. Control rats underwent the same protocol with the control solution containing the same concentration of HP-β-CD and propylene glycol.

Analytical Methods for In Vivo Experiments. Plasma and liver concentrations of ITZ in rats were determined using UPLC-MS/MS system described above. Liver tissue samples were homogenized with phosphate-buffered saline to prepare 20% tissue homogenates and subjected to the same procedure as plasma specimens. Concentrations of biliary lipids were measured by enzymatic methods using Total Bile Acids Test, Phospholipids C Test, and Total Cholesterol Test (Wako Pure Chemicals, Osaka, Japan). Bile specimens were weighed, and the biliary volume was determined assuming a specific gravity of 1.0 g/ml (Fouassier et al., 2002). LFTs (AST, ALT, ALP, γ-GTP, and total bilirubin) in rats were performed by outsourcing service (SRL Tokyo Laboratories, Tokyo, Japan) using plasma specimens collected after 2-h infusion of ITZ. Liver ATP levels after 2-h infusion of ITZ were analyzed according to a conventional method (Tissue ATP Assay Kit; TOYO INK, Tokyo, Japan). Plasma bile acid levels were determined by a method similar to that for the biliary bile acids.

Construction of Recombinant Adenoviruses. Complementary DNA cloning of human MDR3 was performed as described in our previous report (Ikebuchi et al., 2009). For human BSEP, three fragments of the coding region (1–151, 152-2151, and 2152–3969 nucleotides) were amplified from total RNA of HepG2 cells and were ligated by overlapping polymerase chain reaction method to obtain the full-length of BSEP, which was then cloned into HindIII/KpnI sites of pEGFP-N1 vector (Clontech/Takara-Bio, Mountain View, CA). Human Na$^+$/taurocholate cotransporting polypeptide (NTCP/SLC10A1) was amplified from total RNA of HepG2 cells and was cloned into KpnI/XbaI sites of pcDNA3.1/mycHis vector (Invitrogen, Carlsbad, CA).

These cDNAs were then cloned into pTRE-Shuttle2 vectors (MDR3 into NheI/EcoRV sites, BSEP-EGFP into NheI/NotI sites and NTCP-mycHis into SacII/NheI sites) of Adeno-X Tet-Off Expression System 1 Kit (Clontech/Takara-Bio), and recombinant adenoviruses were constructed according to the manufacturer's protocol (Ad-MDR3, Ad-BSEP-EGFP, and Ad-NTCP-mycHis). For mock infections, recombinant adenoviruses containing enhanced green fluorescent protein (EGFP) were similarly constructed (Ad-EGFP). Tet-regulatory adenoviruses (Ad-Tet) required for the expression of target proteins were included in the kit. Those recombinant adenoviruses were amplified in HEK293 cells and purified by the cesium chloride (CsCl) gradient ultracentrifugation method. The viral titers were determined using an Adeno-X Rapid Titer Kit (Clontech/Takara-Bio), and subsequent experiments with adenoviruses were performed after the optimization of multiplicity of infection (MOI) to cells.

MDR3-Mediated Efflux of Phosphatidylcholine. LLC-PK1 cells were seeded on 12-well plates (Falcon; BD Discovery Labware, Bedford, MA) at a density of 1.6×10^5 cells/well, and 48 h later, the cells were infected with 5 MOI Ad-MDR3 and 10 MOI Ad-Tet (LLC-MDR3). For control experiments, Ad-MDR3 was replaced by Ad-EGFP (LLC-EGFP). Twenty-four hours after infection, each well was washed with Krebs-Henseleit buffer (118 mM NaCl, 23.8 mM NaHCO$_3$, 4.83 mM KCl, 0.96 mM KH$_2$PO$_4$, 1.20 mM MgSO$_4$, 12.5 mM HEPES, 5.0 mM glucose, and 1.53 mM CaCl$_2$, pH 7.4) and then preincubated with the buffer containing [^{14}C]choline and drugs (ITZ or verapamil) for 3 h at 37°C under an atmosphere of 5% CO$_2$. After being washed twice with Krebs-Henseleit buffer, the cells were incubated with the buffer containing 3 mM taurocholate and each drug for an additional 3 h. Incubated buffer was collected from each well, lyophilized using a vacuum freeze drier, and diluted in chloroform (medium specimens). The remaining cells were washed twice with ice-cold Krebs-Henseleit buffer, lysed with 0.1 M NaOH plus 0.l% SDS, and then neutralized with 0.1 M HCl. Then, cell lysate was collected from each well, lyophilized, and diluted in chloroform (cell lysate specimens).

The medium and cell lysate specimens were subjected to the thin-layer chromatography on Silica 60 plate (Merck KGaA, Darmstadt, Germany) using a development solvent containing chloroform/methanol/30% ammonia solution 65:35:8. To detect phosphatidylcholine (PC) on the plate, nonradioactive PC was subjected to the plate and visualized using Dragendorff's reagent, which specifically reacts with choline lipids (Wagner et al., 1961). Radioactive PC spots on the thin-layer chromatography plates were detected with a Cyclone Phosphor Imager (PerkinElmer Life and Analytical Sciences), and the cellular efflux activities of PC were evaluated using the following equation: efflux PC/(cellular PC + efflux PC) × 100 (%).

BSEP-Mediated Transport of Taurocholate. LLC-PK1 cells were seeded on Transwell membrane inserts (pore size of 3 μm; BD FALCON) in 24-well plates at a density of 1.4×10^5 cells/insert. Twenty-four hours later, the cells were infected with 10 MOI Ad-BSEP-EGFP, 10 MOI Ad-NTCP-mycHis, and 20 MOI Ad-Tet (LLC-BSEP/NTCP). For control experiments, Ad-BSEP-EGFP was replaced by Ad-EGFP (LLC-EGFP/NTCP). Forty-eight hours after infection, both apical and basal compartments of each well were washed with Krebs-Henseleit buffer and preincubated with ITZ for 3 h at 37°C in an atmosphere of 5% CO$_2$. Thereafter, the basal compartment buffer was substituted with Krebs-Henseleit buffer containing [^{3}H]taurocholate and ITZ. Buffer was collected from each apical compartment at 3 h after incubation, and radioactivity was measured using a Tri-Carb Liquid Scintillation Counter (PerkinElmer Life and Analytical Sciences). At the end of the experiment, the cells were washed twice with Krebs-Henseleit buffer and lysed with 0.1 M NaOH plus 0.l% SDS and then neutralized with 0.1 M HCl. Intracellular radioactivity was similarly measured, and the protein concentration was determined by the method of Lowry (Lowry et al., 1951).

The kinetic parameters of the transcellular transport experiments were calculated according to the method described previously (Mita et al., 2005). For this analysis, the appearance rate of taurocholate in the apical compartment (V_{apical}, in picomoles per hour per milligram of protein) was determined experimentally. Then, the permeability-surface area product (PS) for the basal-to-apical transport of taurocholate (PS$_{\text{b-a}}$, in microliters per hour per milligram of protein) was calculated by dividing V_{apical} by the taurocholate concentration in the basal medium (C_{med}, in picomoles per microliter). In addition, the PS product for the efflux of taurocholate across the apical membrane (PS$_{\text{apical}}$, in microliters per hour per milligram of protein) was calculated by dividing V_{apical} by the taurocholate concentration in cells (C_{cell}, in picomoles per microliter). C_{cell} was calculated assuming that the cellular volume per milligram of cellular protein is 4 μl (Mita et al., 2006).

Western Blot Analyses. LLC-PK1 cells were seeded on 60-mm dishes (BD FALCON) at a density of 1.0×10^6 cells/dish and infected with the adenoviruses at the MOI described in the transport experiments. Crude membrane fractions were prepared, and protein concentrations were determined by the method of Lowry (Lowry et al., 1951). Proteins (15 μg/lane) were size-fractionated on 7% (for MDR3 and BSEP-EGFP) or 10% (for NTCP-mycHis) of SDS-polyacrylamide gels and transferred onto Immobilon membranes (Millipore, Billerica, MA). Molecular weights of proteins were examined using a PageRuler Prestained Protein Ladder (Fermentas, Burlington, ON, Canada). After blocking with 3% bovine serum albumin, membranes were immunoblotted with primary antibodies; mouse P3II26 antibodies (MONOSAN, Uden, the Netherlands) for MDR3, mouse anti-GFP antibodies (Sigma-Aldrich) for BSEP-EGFP, and mouse anti-c-myc antibodies (Roche Diagnostics, Indianapolis, IN) for NTCP-mycHis. As the secondary antibody, anti-mouse IgG antibody labeled with horseradish peroxidase (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) was used. These immunoblotted membranes were treated with an ECL Plus (GE Healthcare) and analyzed using a Chemidoc XRS (Bio-Rad Laboratories, Hercules, CA).

Immunostaining of Transporters. LLC-PK1 cells were seeded on 35-mm glass dishes (IWAKI, Chiba, Japan) at a density of 4.0×10^5 cells/dish and infected with the adenoviruses at the MOI described in the transport experiments. LLC-MDR3 cells were fixed with 100% methanol and immunostained with anti-MDR3 antibody as the primary antibody, and anti-mouse IgG antibody was labeled with Alexa Fluor 488 (Invitrogen) as the secondary antibody. LLC-BSEP/NTCP cells were similarly fixed and immunostained with anti-c-myc antibody followed by anti-mouse IgG antibody labeled with Alexa Fluor 543 (Invitrogen). In each specimen, nuclei were visualized with TO-PRO-3 (Invitrogen). Observations of samples were performed using a FV1000 Confocal Laser Microscope (Olympus, Tokyo, Japan).

Results

Abnormalities in LFTs Were Associated with the Elevated Plasma ITZ Concentration in Patients with DILI. In patients 1, 2, and 3, ITZ may be the cause of liver injury, because elevations in LFTs were observed during the administration of ITZ and LFTs returned to normal after discontinuing ITZ and because possibilities of other common causes of hepatic injury were excluded. Furthermore, in pa-

tients 1 and 2, serum ITZ concentrations were significantly high, which should increase the risk of hepatotoxicity. Patients 1, 2, and 3 suffered from ITZ-induced liver injury 62, 20, and 2 days after starting ITZ-administration, respectively (Table 1), which is consistent with the previous report that the time period to exhibit elevations in LFTs after starting ITZ administration varied from several days to several weeks in ITZ-induced liver injury (Lavrijsen et al., 1992; Hann et al., 1993; Gallardo-Quesada et al., 1995; Srebrnik et al., 2005). In the present study, day 0 was defined as the day on which ITZ administration was started for patients 1 and 2. For patient 3, day 0 was defined as the day from which the serum specimens were obtained, and ITZ administration was started on day 23. In addition, the list of simultaneously administered drugs is shown in Table 1.

Patient 1 experienced elevations in LFTs with ALT 81 IU/l, AST 70 IU/l, ALP 312 IU/l, γ-GTP 80 IU/l, and total bilirubin 0.8 mg/dl on day 70 (Fig. 1, A–C). On day 72, administration of ITZ and other drugs (furosemide, spironolactone, and demethylchlortetracycline) were discontinued, and LFTs gradually returned to normal. Because of the therapeutic requirements, the patient received further ITZ from day 86, and elevations in LFTs were observed again. We found that the trough concentration of serum ITZ reached 1.7 μM (1238 ng/ml) on day 61 (Fig. 1D), which was 2.5-fold higher than the mean trough concentration in a Japanese population receiving the same dose of ITZ (Yamaguchi et al., 2006). Because the peaks of serum ITZ and elevated LFTs were observed at almost the same time on day 70, we have to consider both possibilities that the increase in ITZ concentration caused liver damage and that liver damage caused the increase in ITZ concentration. After readministration from day 86, the serum ITZ concentration increased again and reached 3.0 μM (2131 ng/ml) on day 110. It was found that, although serum concentration of ITZ in patient 1 is higher on day 110 than on day 100, elevated LFTs were more remarkable on day 105 than on day 110. This discrepancy may be accounted for by a hypothesis that the peak concentration of ITZ may have been observed between days 100 and 110, although it is difficult to certify this point because of the absence of serum specimens. Another possibility is that cumulative damage of the liver might cause the decreased metabolism of ITZ and resulted in the increase in its serum concentration.

In patient 2, elevations in LFTs were observed 3 weeks after starting ITZ and famotidine. Although administration of these two drugs was discontinued on day 25, LFTs were still elevated with ALT 874 IU/l, AST 432 IU/l, ALP 539 IU/l, γ-GTP 356 IU/l, and total bilirubin 1.4 mg/dl on day 34 and then gradually returned to normal (Fig. 1, E–G). The serum ITZ concentrations were clearly increased before elevations in LFTs were observed

(Fig. 1, E–G); the trough concentration of serum ITZ reached 2.0 μM (1365 ng/ml) on day 20 (Fig. 1H), which was 2.7-fold higher than the mean trough concentration in a Japanese population (Yamaguchi et al., 2006). These results suggest that the increase in ITZ concentration may have caused liver damage in patient 2. In contrast, the serum concentration of famotidine on day 25 was in a normal range of its clinical use ($<$10 ng/ml as the trough concentration).

In patient 3, although ITZ was suspected to be the cause of liver injury because elevations in LFTs were observed during ITZ administration and LFTs returned to normal after discontinuing the drug, its serum concentration was lower compared with the therapeutic serum concentrations. Simultaneously administered drugs may not be related to the cause of liver injury, because these other drugs were administered continuously during the period. Before ITZ administration, this patient had experienced brotizolam-induced liver injury, and the peak of elevated LFTs were observed on day 7 as ALT, 269 IU/l; AST, 153 IU/l; ALP, 178 IU/l; γ-GTP, 126 IU/l; and total bilirubin, 4.3 mg/dl, which was 2 days after starting brotizolam administration (Fig. 1, I–K). On day 9, brotizolam administration was discontinued and, consequently, on day 17, LFTs returned to normal. On day 23, ITZ administration was started for the patient, and LFTs were elevated again with ALT, 172 IU/l; AST, 135 IU/l; ALP, 343 IU/l; γ-GTP, 524 IU/l; and total bilirubin, 8.6 mg/dl on day 35. The maximum trough concentration of serum ITZ was 0.069 μM (48.9 ng/ml) (Fig. 1L), which was much lower than those in patients 1 and 2, and it is possible that brotizolam-induced liver injury that occurred before ITZ administration might have increased the sensitivity to the cholestatic effect of ITZ.

Effect of ITZ on the Bile Flow Rate and Biliary Secretion of Bile Acids and Phospholipids. ITZ has been reported to cause cholestatic liver injuries (Lavrijsen et al., 1992; Hann et al., 1993; Gallardo-Quesada et al., 1995; Srebrnik et al., 2005), and our clinical data also suggested a cholestatic effect of ITZ with elevations of ALP, γ-GTP, and direct bilirubin (Fig. 1). To characterize the effect of ITZ in in vivo experiments, we analyzed the secretions of biliary lipids in rats whose plasma concentration of ITZ was set at approximately 11 μM, which was confirmed by UPLC-MS/MS. This concentration should correspond to the peak plasma concentration of ITZ in patients 1 and 2; because the peak concentration (C_{max}) is estimated to be 3- to 4-fold higher than the trough concentration after once-daily dosing (Yamaguchi et al., 2006), the C_{max} values of ITZ in the two patients are expected to be as high as 11 μM.

Our in vivo experiments indicated that the bile flow rate was significantly decreased at 30 min after starting ITZ administration (ITZ-treated versus control rats = 55.1 ± 7.4

TABLE 1
Profiles of drug administration to the patients

Patient No.	Age/Sex	Period of ITZ Administration[a]	Time to Elevated LFTs from Starting ITZ[a]	Other Medication
	years		*days*	
1	67/Female	Days 0–71, days 86–110	62	Furosemide, spironolactone, demethylchlortetracycline
2	43/Male	Days 0–24	20	Famotidine
3	36/Female	Days 23–36[b]	2[b]	Cyclosporin A, omeprazole, zolpidem, sulfamethoxazole/trimethoprim, cefepime, vancomycin, ganciclovir, methylprednisolone

[a] See Fig. 1.
[b] Before starting the administration of ITZ, patient 3 received the administration of brotizolam on days 5 to 8 and experienced brotizolam-induced liver injury.
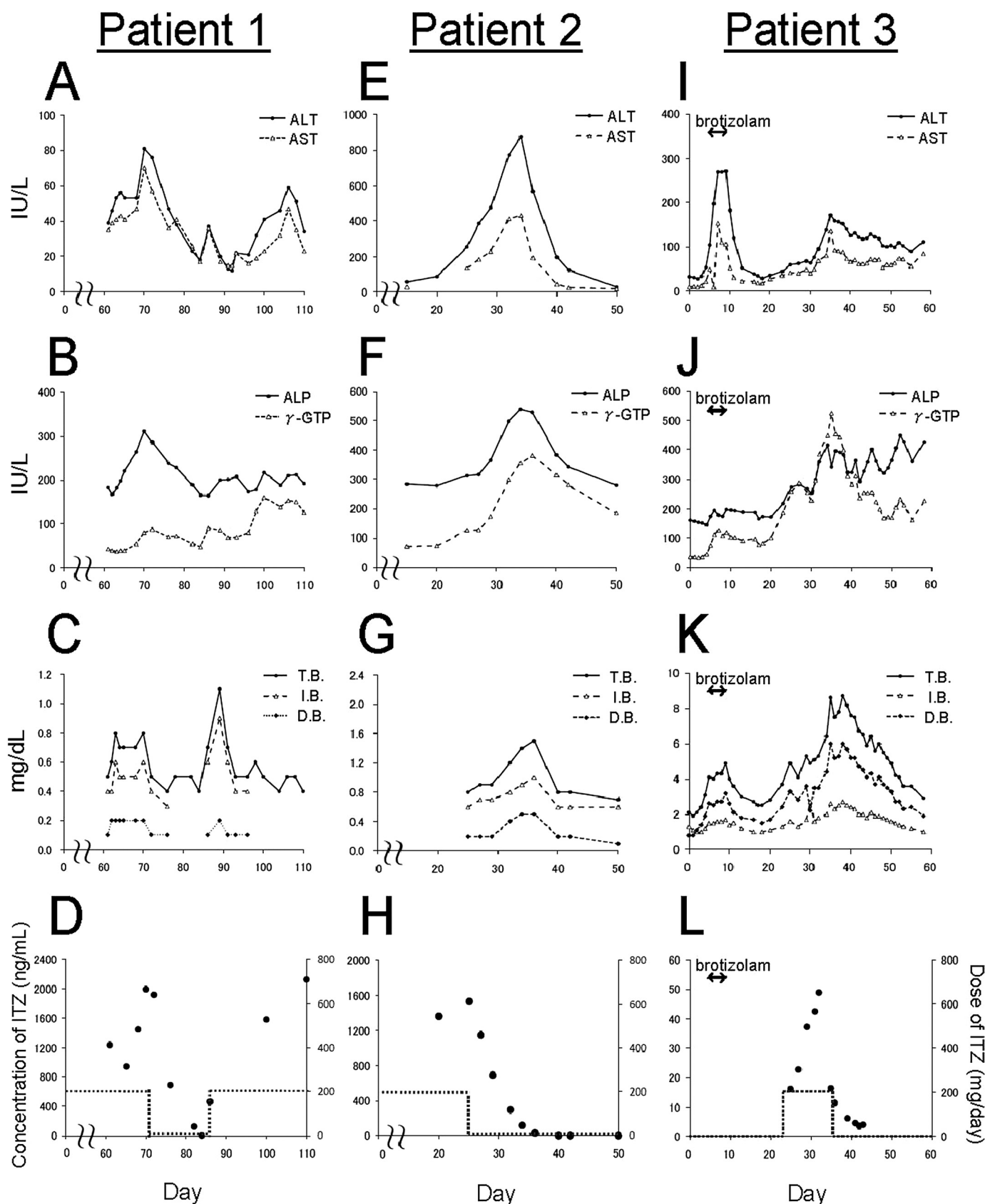
Fig. 1. Profiles of LFTs and serum ITZ concentrations in patients with DILI. Patient 1 (A–D), patient 2 (E–H), and patient 3 (I–L) received ITZ administration and exhibited elevations in LFTs. A, E, and I, serum levels of ALT and AST. B, F, and J, serum levels of ALP and γ-GTP. C, G, and K, serum levels of total bilirubin (T.B.), indirect bilirubin (I.B.), and direct bilirubin (D.B.). D, H, and L, circles represent the serum ITZ concentrations, whereas the dotted lines show the daily dose of ITZ. The arrows in I to L indicate the period of brotizolam administration.

versus 81.2 ± 5.4% of the initial bile flow rate), and this effect continued during the 2-h infusion (Fig. 2A). Although the output of bile acids was decreased in the ITZ administered rats (ITZ-treated versus control rats = 60.6 ± 14.8 versus 85.5 ± 10.0% of the initial secretion) at 30 min, effects were more significant in the phospholipids output (ITZ-treated versus control rats = 49.9 ± 8.3 versus 102.8 ± 3.2% of the initial secretion) and cholesterol output (ITZ-treated versus control rats = 44.8 ± 8.2 versus 96.8 ± 5.4% of the initial secretion) (Fig. 2, B–D). To exclude the possibility that the acute hepatotoxicity of ITZ secondarily caused the changes in biliary components, LFTs (AST, ALT, ALP, γ-GTP, and total bilirubin) and liver ATP levels were analyzed in ITZ-administered rats. It was indicated that no significant changes were observed by the administration of ITZ (Supplemental Table S1 and Supplemental Fig. S1). On the other hand, plasma bile acids were slightly increased in ITZ-administered rats (Supple-

mental Fig. S2), which may be accounted for by considering the secondary effect caused by the reduction in the biliary secretion of bile acids (Fig. 2B). Because the secretions of biliary lipids are known to be mediated by ABC transporters expressed on the bile canalicular membrane, our in vivo data suggest that ITZ has some inhibitory effects on these transporters.

Effect of ITZ on MDR3-Mediated Efflux of PC In Vitro. Some of the drug-induced cholestasis may result from the inhibition of MDR3 and/or BSEP, because the genetic disruption of these transporters causes PFIC diseases. To examine the effect of ITZ on MDR3-mediated transport, we investigated the efflux of PC, a major component of phospholipids in bile (Oude Elferink and Paulusma, 2007), from LLC-MDR3 cells in the presence of ITZ. Results of the Western blot analysis suggested that MDR3 was predominantly expressed as an approximately 140-kDa protein, which may represent the mature and fully glycosylated form (Fig. 3A)
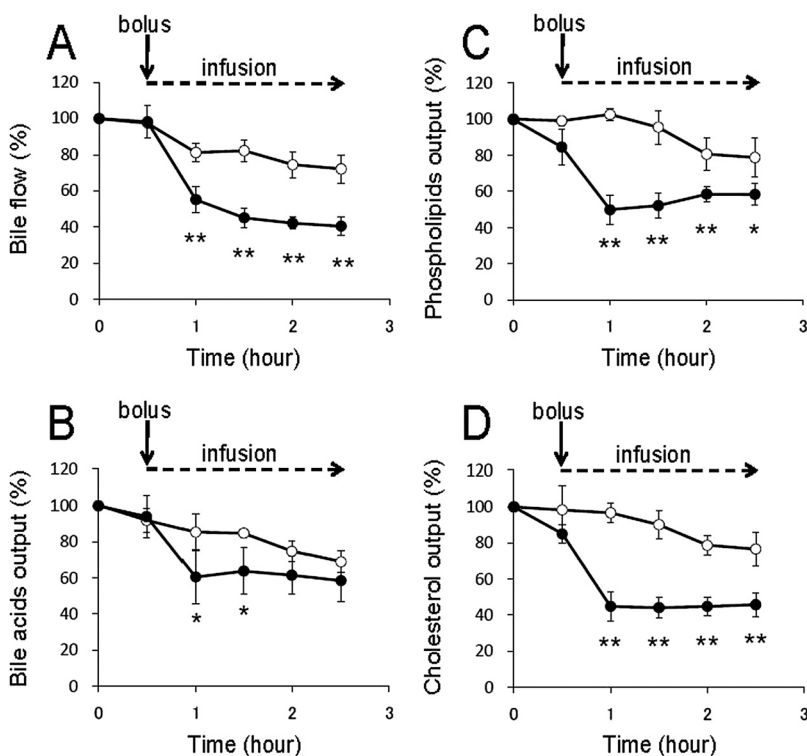

Fig. 2. Effect of ITZ on the bile formation in rats. ITZ (closed symbols) or control solution (open symbols) was administered to rats, and the bile secretion was analyzed. The initial value of each panel is defined as 100%. A, bile flow rate. The initial value was 2.45 ± 0.52 ml/h/kg. B, biliary secretion of bile acids. The initial value was 0.62 ± 0.27 μmol/min/kg. C, biliary secretion of phospholipids. The initial value was 140 ± 52 nmol/min/kg. D, biliary secretion of cholesterol. The initial value was 12.6 ± 3.9 nmol/min/kg. Data are shown as the mean ± S.D. of four independent experiments. ∗∗, $p < 0.01$ and ∗, $p < 0.05$, significantly different from control by Student's t test.
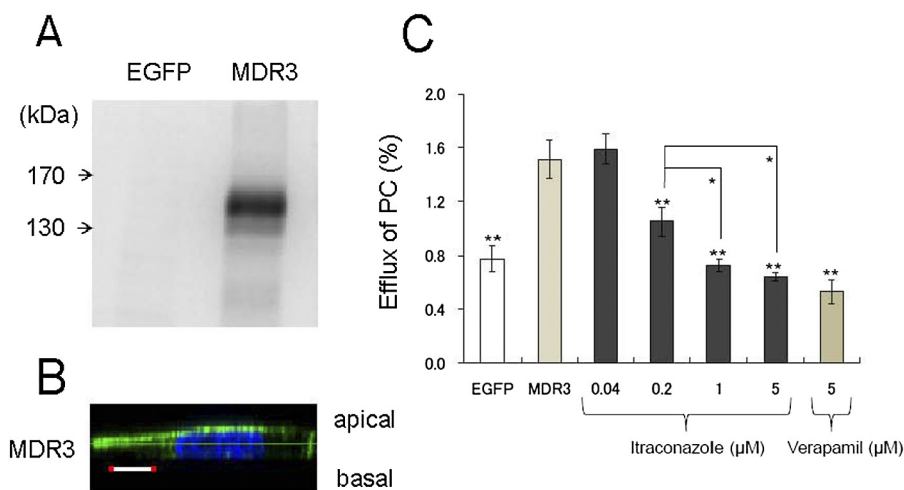

Fig. 3. Effect of ITZ on MDR3-mediated efflux of PC. The expression of MDR3 was determined in LLC-MDR3 cells and the effect of ITZ on MDR3-mediated efflux of PC was examined. A, Western blot analysis was performed to examine the expression of MDR3 protein in LLC-PK1 cells. B, subcellular localization of MDR3 protein was examined by immunofluorescence. The Z-section images are shown. Green and blue fluorescence represent MDR3 and nuclei, respectively. Scale bar, 10 μm. C, efflux of [14C]PC by MDR3-expressing cells was examined at 37°C for 3 h. Cells were incubated with ITZ or verapamil at indicated concentrations. The data are presented as mean ± S.E. ($n = 6$). ∗∗, $p < 0.01$, significantly different from nontreated MDR3-expressing cells by ANOVA followed by Dunnett's test. ∗, $p < 0.05$, significantly different from MDR3-expressing cells treated with 0.2 μM ITZ by ANOVA followed by Student-Newman-Keuls test.

(Morita et al., 2007). In addition, results of immunostaining showed the localization of MDR3 protein on the apical membrane of LLC-PK1 cells (Fig. 3B), which mimic its physiological localization. Because MDR3 is known to mediate the efflux of PC in the presence of lipid acceptors in vitro (Smith et al., 1994; van Helvoort et al., 1996; Morita et al., 2007), we performed PC efflux assay using taurocholate as an acceptor for PC. As shown in Fig. 3C, LLC-MDR3 cells exported twice as much PC as control LLC-EGFP cells, and the MDR3-mediated transport was completely abolished in the presence of 5 μM verapamil, a well established inhibitor of MDR3 (van Helvoort et al., 1996; Morita et al., 2007). Moreover, incubation with 0.04, 0.2, 1, and 5 μM ITZ caused a concentration-dependent increase in the cellular concentration of ITZ confirmed by UPLC-MS/MS (data not shown). In addition, 0.2, 1, and 5 μM ITZ inhibited the function of MDR3 in a concentration-dependent manner (Fig. 3C). This concentration range of ITZ, which exhibited the inhibitory effects on MDR3, should correspond to the in vivo experiments using rats (Fig. 2), because the unbound concentration of ITZ in rat liver was

calculated to be 0.29 μM; this value was calculated by multiplying the ITZ concentration in rat liver in the present analysis (119 $\pm$ 14 μM) by the unbound fraction of ITZ in rat liver (0.0024) reported by Yamano et al. (1999). Together, the inhibition of MDR3 function in vitro and the disturbance of biliary phospholipids secretion in vivo were observed over a similar range of ITZ concentrations, suggesting that ITZ may be involved in the cholestatic liver injury via the inhibition of MDR3-mediated biliary secretion of phospholipids.

Effect of ITZ on BSEP-Mediated Efflux of Taurocholate In Vitro. Furthermore, we examined the effect of ITZ on BSEP function using LLC-BSEP/NTCP cells. The expressions of BSEP fused with EGFP (approximately 180 kDa of protein) and NTCP (approximately 50 kDa of protein), which is an uptake transporter of bile acids (Boyer et al., 1994), were confirmed by the Western blot analyses (Fig. 4A). In addition, the results of immunostaining studies indicated that BSEP and NTCP are expressed on the apical and basal membranes of LLC-BSEP/NTCP cells, respectively, which correspond to the expression under physiological conditions
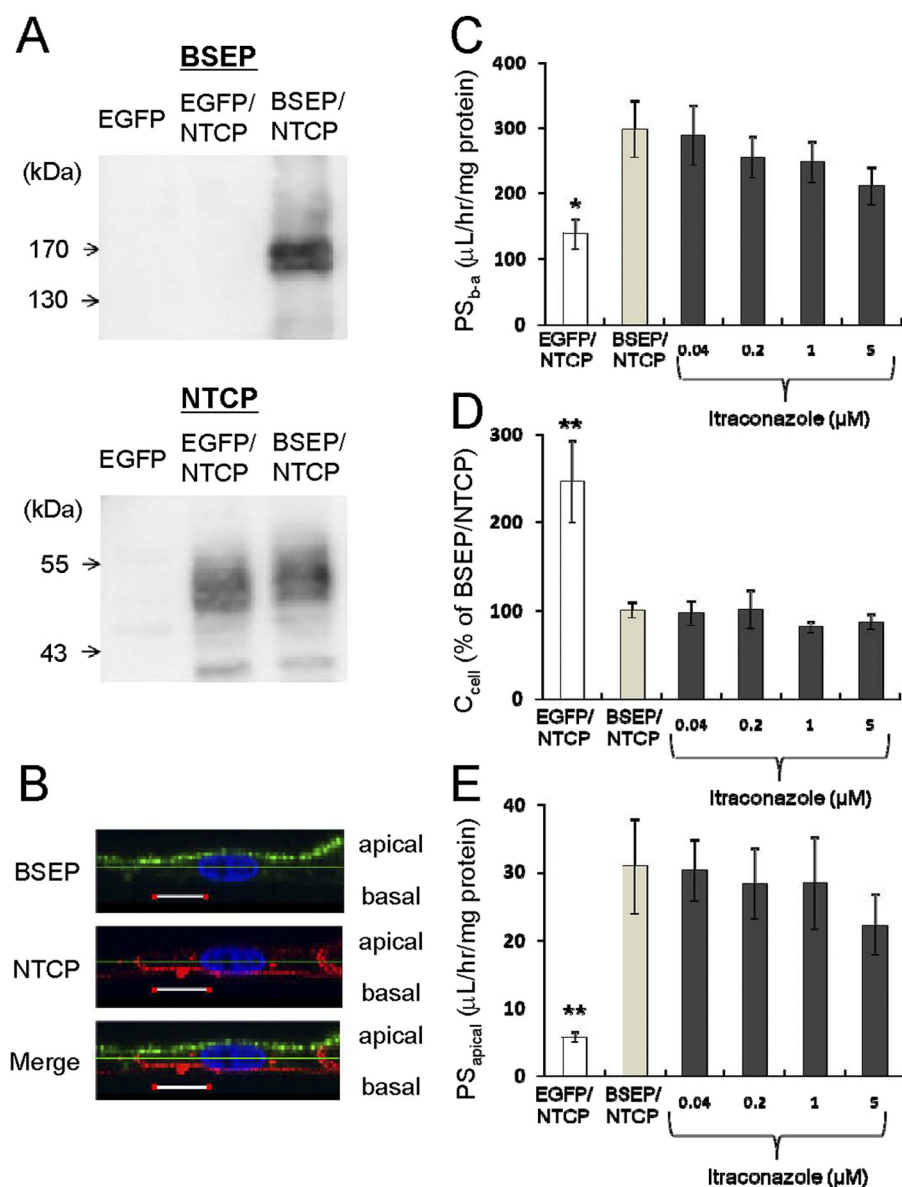

Fig. 4. Effect of ITZ on BSEP/NTCP-mediated transcellular transport of taurocholate. The expression of BSEP/NTCP was determined in the LLC-BSEP/NTCP cells, and the effect of ITZ on BSEP/NTCP-mediated transport of taurocholate was examined. A, Western blot analysis was performed to examine the expression of BSEP and NTCP protein in LLC-PK1 cells. B, subcellular localization of BSEP and NTCP protein was examined by immunofluorescence. The Z-section images are shown. Green, red, and blue fluorescence represent BSEP, NTCP, and nuclei, respectively. Scale bars, 10 μm. C to E, transport of [³H]taurocholate by NTCP- and BSEP/NTCP-expressing cells was examined at 37°C for 3 h. Cells were incubated with ITZ at indicated concentrations. PS_{b-a} of [³H]taurocholate (C), C_{cell} of [³H]taurocholate (D), and PS_{apical} of [³H]taurocholate (E) are shown. The data are presented as mean $\pm$ S.E. ($n = 7$). **, $p < 0.01$, and *, $p < 0.05$, significantly different from nontreated BSEP/NTCP-expressing cells by ANOVA followed by Dunnett's test.

(Fig. 4B). The effect of ITZ on the transcellular transport of taurocholate was examined in LLC-BSEP/NTCP cells, after confirming that the cellular concentrations of ITZ were almost the same as those in LLC-MDR3 cells. It was found that the basal-to-apical transport of taurocholate (PS_{b-a}) across LLC-BSEP/NTCP was not significantly affected by ITZ (Fig. 4C). Considering that the cellular concentration (C_{cell}) and apical membrane-mediated transport of taurocholate (PS_{apical}) were not significantly affected by 0.04 to 5 μM ITZ (Fig. 4, D and E), the effect of ITZ on both BSEP-mediated excretion and NTCP-mediated uptake of bile acids seemed to be minor, if any.

Together, these in vitro results indicated that ITZ inhibits MDR3 function at much lower concentrations (Fig. 3C) compared with those required to inhibit BSEP function (Fig. 4E), which may explain the in vivo observations that ITZ had more significant influence on the biliary secretion of phospholipids than that of bile acids in rats.

Discussion

In the present study, we found two patients with ITZ-induced cholestatic liver injury whose serum ITZ concentrations were increased 3- to 4-fold compared with the normal range in clinical situations (Fig. 1, D and H, patients 1 and 2) (Yamaguchi et al., 2006). Based on these results, we conducted in vivo and in vitro studies to examine the effect of ITZ on biliary secretion. It was found that the biliary secretion of phospholipids, rather than bile acids, was markedly reduced in ITZ-administered rats (Fig. 2), and ITZ was shown to inhibit MDR3-mediated efflux of PC in the in vitro assays (Fig. 3) without significantly affecting BSEP-mediated apical transport of taurocholate (Fig. 4).

According to the previous reports, ITZ-induced liver injury was associated with significant elevations of ALP, γ-GTP, and total bilirubin, which suggest the cholestatic effect of ITZ (Lavrijsen et al., 1992; Hann et al., 1993; Gallardo-Quesada et al., 1995; Srebrnik et al., 2005). In addition, the liver biopsy specimens from patients with ITZ-induced liver injury showed histological patterns of cholestasis (Adriaenssens et al., 2001). In the present study, in patients 1, 2, and 3, ALP, γ-GTP, and total bilirubin were increased during ITZ-administration (Fig. 1), suggesting that cholestasis might be involved in the acquisition and aggravation of those liver injuries. However, ALT and AST levels, which are regarded as the markers of hepatocellular damage, were also elevated, particularly in patient 2. These results suggest the possibility that ITZ causes liver injuries not only by the cholestatic mechanisms but also by direct damage to hepatocytes.

It was found that the serum ITZ concentrations in patients 1 and 2 were much higher than the mean concentrations in Japanese patients (Yamaguchi et al., 2006). The reasons for such increase in ITZ concentrations are unclear; drug-drug interactions between ITZ and the coadministered drugs via its metabolic pathway are not reported, because ITZ is supposed to be predominantly metabolized by CYP3A4 (Nivoix et al., 2008), and none of the coadministered drugs are reported to inhibit this enzyme. In addition, drug-drug interactions via the inhibition of efflux transporters may be unlikely, because ITZ is predominantly eliminated from the body by metabolic conversion. Therefore, we could not attribute the increases in serum ITZ concentrations in patients 1 and 2 to some drug-drug interactions.

To examine the possibility that patients 1, 2, and 3 had some genetic factors in *MDR3* gene to increase the risk of ITZ-induced cholestasis, SNPs in the gene were analyzed by DMET Plus DNA microarray using the patients' genomic specimens. According to the analyses, some alleles that are minor in the Asian population were found in *MDR3* genes of patients 1 and 2 (rs2109505, rs2302387, rs4148808, and rs4148805) (Supplemental Table S2). Among them, two SNPs are silent mutations in the coding region (rs2109505 and rs2302387), and the others are in the upstream region (rs4148808 and rs4148805). Although there has been no report analyzing the effects of these SNPs, it is still possible that they may affect the expression level of MDR3 protein. In addition, SNPs in the *CYP3A4* gene, which might affect the metabolism of ITZ, were analyzed; all patients carried *CYP3A4*1/*1* (wild-type) alleles.

To examine the effect of ITZ under in vivo conditions, rats were administered ITZ and exhibited a significant reduction in the secretion of phospholipids rather than bile acids (Fig. 2). Because these in vivo experiments were performed in the range of ITZ concentration similar to those in patients 1 and 2 (Fig. 1), the inhibition of the secretion of biliary phospholipids may also occur under clinical situations. Elevations in LFTs (AST, ALT, ALP, γ-GTP, and total bilirubin) were not observed after the 2-h infusion of ITZ into rats (Supplemental Table S1), suggesting that the acute liver damage might not take place in the in vivo experiments. In the same manner, Somchit et al. (2004) reported that elevations in LFTs and histological changes (hepatocellular necrosis, degeneration of periacinar and mizonal hepatocytes, bile duct hyperplasia and biliary cirrhosis, and giant cell granuloma) were observed in rats with repeated administration of ITZ but not with a single administration of ITZ (Somchit et al., 2004). Continuous inhibition of MDR3-mediated biliary secretion of phospholipids by ITZ may result in the acquisition of liver injury.

To reveal the inhibitory effect of ITZ on MDR3-mediated transport, in vitro studies were performed using MDR3-expressing LLC-PK1 cells (Fig. 3C). ITZ significantly inhibited the MDR3-mediated efflux of PC at 0.2 μM, which was similar to the intrahepatic unbound concentration in our in vivo experiments (0.29 μM, see *Results*). In contrast, the inhibitory effect of ITZ on the BSEP-mediated apical transport of taurocholate seemed to be weak (Fig. 4E). Together, our in vitro data suggest that ITZ is involved in the acquisition of cholestasis via the inhibition of MDR3 rather than BSEP.

Considering the results of our in vivo data in humans and rats and the in vitro data, it is possible that the inhibitory effect of ITZ on MDR3-mediated biliary phospholipids secretion contributes to the development of cholestasis. Although delayed elevations in LFTs in patients 1 and 2 were observed several weeks after starting ITZ administration, such delays may be explained by hypothesizing that the damage to the bile canalicular membrane via the inhibition of MDR3 acts as one of the factors for the progression of liver injury, which is gradually reflected to abnormal LFTs. In addition, other mechanisms, such as cellular stress and activation of immune system (Gunawan and Kaplowitz, 2007), are also supposed to play a role in clinical situations.

Because both MDR3 and BSEP play essential roles in the process of bile formation, inhibition of their functions may be related to cholestasis induced by not only ITZ but also other

clinically used drugs. At present, little is known about the inhibition of MDR3 by cholestatic drugs, although several drugs have been reported to inhibit BSEP function. For example, troglitazone, which was withdrawn from the market because of its hepatotoxicity, and its sulfate metabolite were shown to inhibit rat BSEP in vitro at concentrations similar to the intrahepatic concentration in a troglitazone-induced cholestatic rat model (Funk et al., 2001). In addition, some other cholestatic drugs such as cyclosporine A, rifampicin, glibenclamide, and chlorpromazine are reported to have inhibitory effects on BSEP in vitro (Stieger et al., 2000; Byrne et al., 2002; Horikawa et al., 2003; Wang et al., 2003), although their K_i or IC_{50} values are much higher than those observed under in vivo conditions. Taking into consideration the fact that γ-GTP, a cholestatic marker, is elevated in MDR3-deficient type PFIC (PFIC3) but not in BSEP-deficient type (PFIC2) in general (Oude Elferink et al., 2006), it is possible that these drugs may inhibit MDR3 at clinically relevant K_i or IC_{50} values, which is lower than for BSEP inhibition.

Although little information is available for drugs causing MDR3 inhibition, there are some candidate drugs. Because MDR3/ABCB4 has the closest similarity to MDR1/ABCB1 among genes in the ABC transporter family and is known to have some overlapping substrate specificities with MDR1, such as digoxin, paclitaxel, and vinblastine (Smith et al., 2000), cholestatic drugs that can act as MDR1 inhibitors may also inhibit MDR3-mediated biliary secretion of phospholipids. In fact, ITZ is a potential inhibitor of MDR1 (Gupta et al., 1991; Takara et al., 1999; Iida et al., 2001) and MDR3. Furthermore, tacrine, which often increases LFTs, may also have an inhibitory effect on MDR3; its risk of hepatotoxicity was particularly high in humans carrying some variants of the *MDR3* gene (Alfirevic et al., 2007). The in vitro experimental system used in the present study may be useful in determining the inhibitory effect of various compounds on MDR3 function.

In conclusion, the results of the present study suggest that the inhibition of MDR3-mediated biliary secretion of phospholipids by ITZ may contribute to its cholestatic effect. The possibility of MDR3 inhibition, in addition to BSEP inhibition, should be taken into account for a better understanding of the mechanism of drug-induced cholestasis. The in vitro cell systems constructed in the present study should be useful for evaluating cholestatic potential of clinically used drugs and drug candidates under development.

Authorship Contributions

Participated in research design: Yoshikado, Takada, Yamamoto, Ito, Yokota, Yatomi, Yoshida, Goto, Tsuji, and Suzuki.

Conducted experiments Yoshikado and Yamamoto.

Contributed new reagents or analytic tools: Yamaji and Ito.

Performed data analysis: Yoshikado, Takada, Yamamoto, Santa, and Suzuki.

Wrote or contributed to the writing of the manuscript: Yoshikado, Takada, and Suzuki.

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